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Novel Isoflavone Glucosides in Groundnut (*Apios americana* Medik) and Their Antiandrogenic Activities

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ABSTRACT: Isoflavone glucosides (2′-hydroxy,5-methoxy genistein-7-*O*-glucoside (**1**), 2′-hydroxy genistein-7-*O*-gentibioside (**2**), 5-methoxy genistein-7-*O*-glucoside (**3**), 3′,5-dimethoxy genistein-7-*O*-glucoside (**4**), 2′-hydroxy genistein-7-*O*-glucoside (**5**), genistein-7-*O*-gentibioside (**6**), 2′-hydroxy,5-methoxy genistein-4′,7-*O*-diglucoside (**7**), and 2′-hydroxy genistein-4′,7-*O*-diglucoside (**8**)) were isolated from the groundnut of *Apios americana* Medik. Their structures were elucidated on the basis of HR-ESI-MS and 1D- and 2D-NMR analyses. Compounds **1**, **2**, **4**, and **7** are new compounds presented here for the first time. Compounds **2** and **5** were proven to be androgen receptor antagonists due to their binding activities for androgen receptors (IC_{50} 280 and 160 μM, respectively) and the inhibitory activity of androgen-induced expression of prostate-specific antigen (PSA) mRNA in LNCaP (prostate adenocarcinoma) cells (IC_{50} 20 and 18 μM, respectively).

KEYWORDS: *Apios americana Medik, isoflavone glucosides, antiandrogenic activity, LNCaP human prostate adenocarcinoma cells*

INTRODUCTION

Groundnut (*Apios americana* Medik) is a leguminous perennial vine native to North America that generates edible tubers, which were used as an important food by native Americans. This plant is called hodoimo or America-hodoimo in Japan, and its tubers have also been eaten in Japan, specifically in Aomori Prefecture, for more than one hundred years. This food is believed to be very nutritious, especially for women just after childbirth. Several studies have reported on the fatty acid,[1] amino acid,[2] and carbohydrate[3] compositions in groundnut, while other studies have reported on its secondary metabolites (saponin,[4] genistein,[5] and genistein glucosides[6]).

Since many isoflavones have been shown to possess interesting biological activities,[7−9] we analyzed the extract of the groundnut using PDA HPLC to obtain new bioactive isoflavones and found 7 isoflavone glucosides that have not been reported previously (Figure 1). In this study, we isolated all of these compounds using chromatographic methods and determined their structures (4 compounds were novel).

In addition, because some isoflavones such as genistein and daidzein were reported to show an estrogen agonist ability,[10] we tested the receptor binding activities of the isoflavone glucosides for the estrogen receptor (ER) and androgen receptor (AR) (androgen is structurally similar to estrogen). Two compounds were clarified to possess binding affinity for AR, while none of the compounds possessed binding affinity for ER. The activity of the 2 compounds for AR was proven to be antagonistic with an assay using LNCaP human prostate adenocarcinoma cells.

MATERIALS AND METHODS

Sample Materials. Groundnut (*Apios americana* Medik) was purchased from e-yakusou.com (Niigata, Japan; http://www.e-yakusou.com/nae/apos.html) and planted in Ishikawa Prefectural Universiy in 2011, and the harvested tubers were used in this study. Gensitein, daidzein, and flutamide were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Isolation of Isoflavone Glucosides from Groundnut. Freeze-dried groundnuts (261.9 g) were pulverized in a mixer, and extracted with 2 L of MeOH by stirring for 30 min at room temperature (×2). The extract was filtered, and the filtrate was concentrated to dryness in vacuo to give a yellowish oil (1.02 g). This oil was added to 100 mL of hexane and sonicated for 5 min. The insoluble precipitate (1.02 g) was collected by centrifugation and applied on a silica gel column [30 i.d. × 200 mm, Silica Gel60 (Kanto Chemical Co. Inc., Tokyo, Japan)] prepared and developed with CH_2Cl_2:MeOH:H_2O (3:1:0.1). Fractions 15−70 were collected and concentrated to dryness to give a pale yellowish oil (0.26 g). This oil was analyzed by RP-HPLC chromatography using Developsil C30 (20 i.d. × 250 mm; Nomura Chemical Co. Ltd., Aichi, Japan) developed with 17% CH_3CN (flow rate: 7.0 mL/min). From this, pure compounds **1**, **2**, **3**, **4**, **5**, and **6** and crude compounds **7** and **8** were obtained. Crude compounds **7** and **8** were both further purified by ODS HPLC using CAPCELLPAK SG (10 i.d. × 250 mm; Shiseido, Tokyo, Japan) developed with 20% MeOH (flow rate: 3.0 mL/min) to give pure compounds.

Spectroscopic Analysis. NMR spectra were measured on AVANCE400 (Bruker BioSpin, Karlsruhe, Germany) in DMSO-d_6 with the residual solvent peak as an internal standard (δ_C 39.5, δ_H 2.50 ppm) for **1**, **3**, **4**, **5**, **7**, and **8** or CD_3OD (δ_C 49.5, δ_H 3.30 ppm) for **2**,

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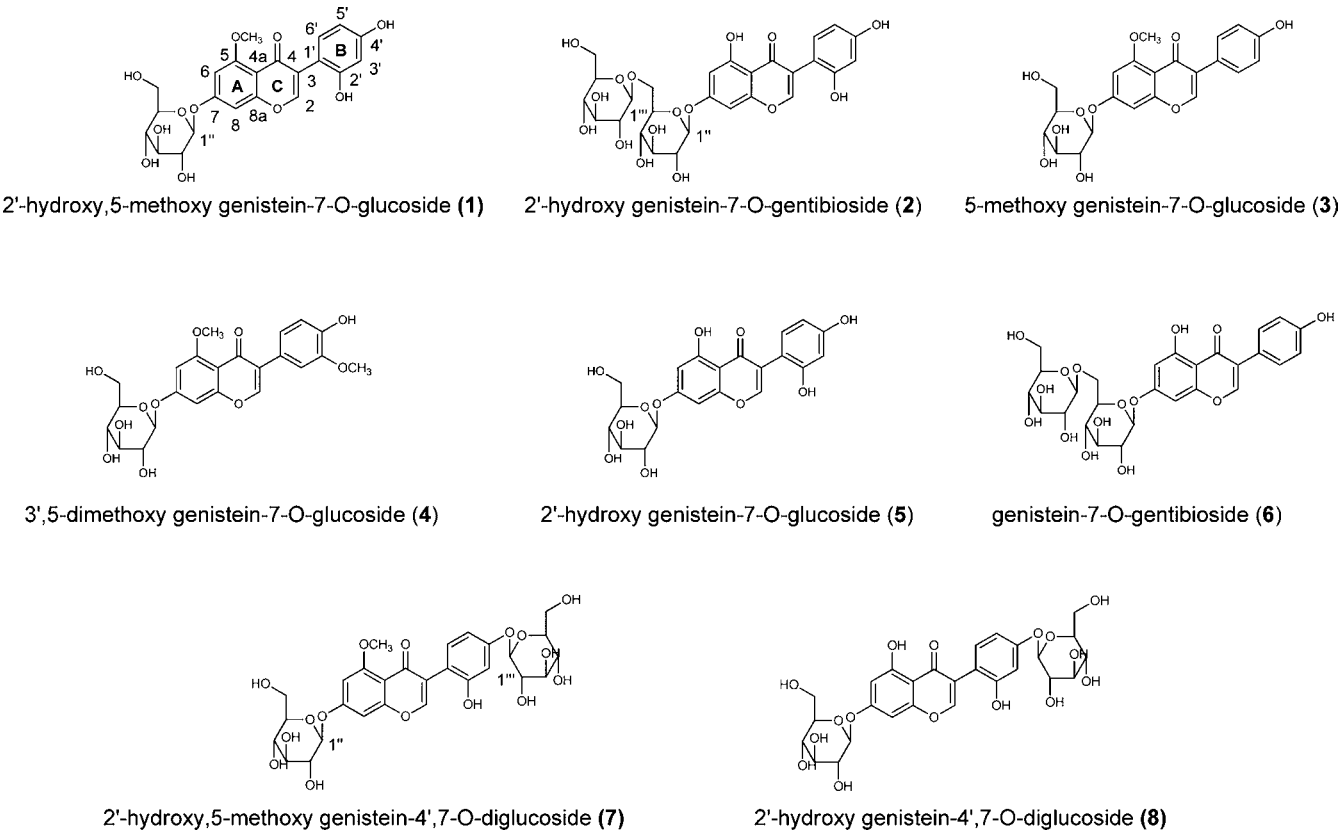

Figure 1. The structures of isoflavone glucosides.

pyridine-d_5 (δ_C 135.5, δ_H 7.58 ppm) for **6**, or $CDCl_3$ (δ_C 77.0, δ_H 7.25 ppm) for hexa-acetyl **1**. HR-ESI-MS was recorded with JMS-T100LP (Jeol, Tokyo, Japan) using reserpine as an external standard.

Physico-Chemical Data for Compounds 1 − 8 and Hexa-acetyl 1. *2′-Hydroxy,5-methoxy genistein-7-O-glucoside (1).* White solid; $[\alpha]_D^{22}$ −72.0° (*c* 0.1, 50%MeOH), UV(MeOH) λ_{max} 256 nm. HR-ESI-MS (negative) *m*/*z* 461.10893 $[M - H]^-$ (calcd for $C_{22}H_{21}O_{11}$ 461.10839). ^{1}H NMR (DMSO-d_6) δ: 3.16 (m, 1H, H-4′′), 3.30 (m, 1H, H-2′′), 3.36 (m, 1H, H-3′′), 3.45 (2H, H-5′′ and H-6′′b), 3.72 (m, 1H, H-6′′a), 3.83 (s, 3H, H-5OCH$_3$), 5.08 (d, *J* = 7.2 Hz, 1H, H-1′′), 6.25 (dd, *J* = 2.0, 8.2 Hz, 1H, H-5′), 6.34 (d, *J* = 2.0 Hz, 1H, H-3′), 6.60 (d, *J* = 2.0 Hz, 1H, H-6), 6.72 (d, *J* = 2.0 Hz, 1H, H-8), 6.91 (d, *J* = 8.2 Hz, 1H, H-6′), 8.01 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6) δ: 56.1 (C-5OCH$_3$), 60.7 (C-6′′), 69.8 (C-4′′), 73.1 (C-3′′), 76.6 (C-2′′), 77.3 (C-5′′), 95.6 (C-8), 97.1 (C-6), 99.1 (C-1′′), 102.8 (C-3′), 106.2 (C-5′), 109.5 (C-4a), 110.3 (C-1′), 123.3 (C-3), 132.1 (C-6′), 152.1 (C-2), 156.4 (C-2′), 158.3 (C-4′), 158.8 (C-8a), 160.6 (C-5), 161.3 (C-7), 174.4 (C-4).

Hexacetyl 1. White solid; HR-ESI-MS (negative) *m*/*z* 713.17195 $[M - H]^-$ (calcd. for $C_{34}H_{33}O_{17}$ 713.17177). ^{1}H NMR ($CDCl_3$) δ: 2.06 − 2.13 (CH$_3$COOH × 6), 3.92 (s, 3H, C-5OCH$_3$), 3.96 (ddd, *J* = 2.3, 5.6, 9.0 Hz, 1H, H-5′′), 4.22 (dd, *J* = 2.3, 12.3 Hz, 1H, H-6′′b), 4.28 (dd, *J* = 5.6, 12.3 Hz, 1H, H-6′′a), 5.18 (dd, *J* = 9.0, 9.3 Hz, 1H, H-4′′), 5.24 (d, *J* = 7.2 Hz, 1H, H-1′′), 5.31 (dd, *J* = 7.2, 9.0 Hz, 1H, H-2′′), 5.35 (dd, *J* = 9.0, 9.3 Hz, 1H, H-3′′), 6.45 (d, *J* = 2.0 Hz, 1H, H-6), 6.58 (d, *J* = 2.0 Hz, 1H, H-8), 7.03 (d, *J* = 2.2 Hz, 1H, H-3′), 7.06 (dd, *J* = 2.2, 8.3 Hz, H-5′), 7.35 (d, *J* = 8.3 Hz, H-6′), 7.70 (s, 1H, H-2). ^{13}C NMR ($CDCl_3$) δ: 20.6 − 20.9 (CH$_3$COOH × 6), 56.5 (C-5OCH$_3$), 62.0 (C-6′′), 68.1 (C-4′′), 71.0 (C-2′′), 72.5 (C-3′′), 72.5 (C-5′′), 95.8 (C-8), 97.6 (C-6), 98.0 (C-1′′), 111.1 (C-4a), 116.4 (C-3′), 119.0 (C-5′), 122.4 (C-1′), 123.0 (C-3), 132.1 (C-6′), 149.4 (C-2′), 151.1 (C-4′), 151.7 (C-2), 159.4 (C-8a), 160.6 (C-7), 161.7 (C-5), 168.8 − 170.4 (CH$_3$COOH × 6), 174.0 (C-4).

2′-Hydroxy Genistein-7-O-gentibioside (2). White solid; $[\alpha]_D^{22}$ −53.2° (*c* 0.5, MeOH), UV(MeOH) λ_{max} 260 nm. HR-ESI-MS (negative) *m*/*z* 609.14474 $[M - H]^-$ (calcd for $C_{27}H_{29}O_{16}$ 609.14556). ^{1}H NMR (CD_3OD) δ: 3.23 (2H, H-2‴ and H-5‴), 3.27 (m, 1H, H-3‴), 3.33 (m, 1H, H-4′′), 3.47 (m, 1H, H-3′′), 3.49 (m, 1H, H-2′′), 3.67 (dd, *J* = 6.2, 11.8 Hz, 1H, H-6‴b), 3.84 (m, 1H, H-6′′b), 3.85 (m, 1H, H-5′′), 3.89 (d, *J* = 11.8 Hz, 1H, H-6‴a), 4.16 (d, *J* = 9.0 Hz, 1H, H-6′′a), 4.37 (d, *J* = 7.2 Hz, 1H, H-1‴), 5.05 (d, *J* = 7.2 Hz, 1H, H-1′′), 6.37 (dd, *J* = 2.0, 8.1 Hz, 1H, H-5′), 6.57 (d, *J* = 2.0 Hz, 1H, H-6), 6.57 (d, *J* = 2.0 Hz, 1H, H-3′), 6.87 (d, *J* = 2.0 Hz, H-8), 7.05 (d, *J* = 8.1 Hz, 1H, H-5′), 8.11 (s, 1H, H-2). ^{13}C NMR (CD_3OD) δ: 62.8 (C-6‴), 70.5 (C-6′′), 71.6 (C-4′′)[a], 71.7 (C-4‴)[a], 74.7 (C-3′′), 75.2 (C-3‴), 77.3 (C-5′′), 77.8 (C-2′′), 78.0 (C-5‴), 78.1 (C-2‴), 96.0 (C-8), 101.3 (C-6), 101.5 (C-1′′), 104.2 (C-3′), 105.2 (C-1‴), 108.1 (C-4a), 108.1 (C-5′), 110.6 (C-1′), 122.9 (C-3), 133.3 (C-6′), 157.3 (C-2), 157.8 (C-2′), 159.4 (C-8a), 160.3 (C-4′),163.3 (C-5), 164.7 (C-7), 182.9 (C-4). [a]: interchangeable.

5-Methoxy Genistein-7-O-glucoside (3). White solid; $[\alpha]_D^{22}$ −44.3° (*c* 0.5, MeOH), UV(MeOH) λ_{max} 257 nm. HR-ESI-MS (negative) *m*/*z* 445.11528 $[M - H]^-$ (calcd for $C_{22}H_{21}O_{10}$ 445.11347). ^{1}H NMR (DMSO-d_6) δ: 3.16 (m, 1H, H-4′′), 3.30 (m, 1H, H-2′′), 3.32 (m, 1H, H-3′′), 3.45 (2H, H-5′′ and H-6′b), 3.74 (m, 1H, H-6′′a), 3.84 (s, 3H, C-5OCH$_3$), 5.08 (d, *J* = 7.1 Hz, 1H, H-1′′), 6.60 (d, *J* = 2.0 Hz, 1H, H-6), 6.73 (d, *J* = 2.0 Hz, 1H, H-8), 6.79 (d, *J* = 8.4 Hz, 2H, H-3′ and H-5′), 7.31 (d, *J* = 8.4 Hz, 2H, H-2′ and H-6′), 8.16 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6) δ: 56.1 (C-5OCH$_3$), 60.7 (C-6′′), 69.8 (C-4′′), 73.1 (C-2′′), 76.6 (C-3′′), 77.3 (C-5′′), 95.6 (C-8), 97.1 (C-6), 99.9 (C-1′′), 109.5 (C-4a), 114.8 (C-3′ and C-5′), 122.6 (C-1′), 124.9 (C-3), 131.5 (C-2′ and C-6′), 150.8 (C-2), 157.2 (C-4′), 158.8 (C-8a), 160.8 (C-5), 161.3 (C-7), 173.9 (C-4).

3′,5-Dimethoxy Genistein-7-O-glucoside (4). White solid; $[\alpha]_D^{22}$ −48.4° (*c* 0.5, MeOH), UV(MeOH) λ_{max} 259 nm. HR-ESI-MS (negative) *m*/*z* 475.12413 $[M - H]^-$ (calcd for $C_{23}H_{23}O_{11}$ 475.12404). ^{1}H NMR (DMSO-d_6) δ: 3.16 (m, 1H, H-4′′), 3.30 (2H, H-2′′ and H-3′′), 3.45 (2H, H-5′′ and H-6′′b), 3.72 (m, 1H, H-6′′a), 3.78 (s, 3H, C-3′′OCH$_3$), 3.84 (s, 3H, C-5OCH$_3$), 5.08 (d, *J* = 7.2 Hz, 1H, H-1′′), 6.60 (s, 1H, H-6), 6.73 (s, 1H, H-8), 6.79 (d, *J* = 8.2 Hz, 1H, H-5′), 6.90 (d, *J* = 8.2 Hz, 1H, H-6′), 7.11 (s, 1H, H-2′), 8.19 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6) δ: 55.6 (C-3′OCH$_3$), 56.1

(C-5O<u>C</u>H$_3$), 60.7 (C-6″), 69.7 (C-4″), 73.1 (C-3″), 76.6 (C-2″), 77.3 (C-5″), 95.6 (C-8), 97.1 (C-6), 99.8 (C-1″), 109.5 (C-4a), 113.4 (C-2′), 115.1 (C-5′), 121.6 (C-6′), 123.0 (C-1′), 124.9 (C-3), 146.4 (C-4′), 147.0 (C-3′), 151.1 (C-2), 158.7 (C-8a), 160.8 (C-5), 161.3 (C-7), 173.8 (C-4).

*2′-Hydroxy Genistein-7-O-glucoside (**5**).* White solid; $[\alpha]_D^{22}$ −58.8° (*c* 0.5, MeOH)UV(MeOH) λ_{max} 260 nm. HR-ESI-MS (negative) *m*/*z* 447.09314 $[M - H]^-$ (calcd for $C_{21}H_{19}O_{11}$ 447.09274). ^{1}H NMR (DMSO-d_6) δ: 3.16 (m, 1H, H-4″), 3.29 (m, 1H, H-2″), 3.30 (m, 1H, H-3″), 3.44 (m, 1H, H-5″), 3.46 (m, 1H, H-6″b), 3.70 (m, 1H, H-6″a), 5.06 (d, *J* = 7.4 Hz, 1H, H-1″), 6.27 (d, *J* = 8.1 Hz, 1H, H-5′), 6.38 (s, 1H, H-3′), 6.46 (s, 1H, H-6), 6.70 (s, 1H, H-8), 6.99 (d, *J* = 8.1 Hz, 1H, H-6′), 8.26 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6) δ: 60.6 (C-6″), 69.6 (C-4″), 73.1 (C-3″), 76.4 (C-2″), 77.2 (C-5″), 94.4 (C-8), 99.5 (C-6), 99.9 (C-1″), 102.6 (C-3′), 106.1 (C-4a), 106.2 (C-5′), 108.3 (C-1′), 120.8 (C-3), 132.2 (C-6′), 155.9 (C-2), 156.5 (C-2′), 157.2 (C-8a), 158.7 (C-4′), 161.6 (C-5), 162.9 (C-7), 180.7 (C-4).

*Genistein-7-O-gentibioside (**6**).* White solid; $[\alpha]_D^{22}$ −62.4° (*c* 0.5, MeOH)UV(MeOH) λ_{max} 262 nm. HR-ESI-MS (negative) *m*/*z* 593.15152 $[M - H]^-$ (calcd. for $C_{27}H_{29}O_{15}$ 593.15064). ^{1}H NMR (pyridine-d_5) δ: 3.92 (m, 1H, H-5‴), 4.22 (m, 1H, H-4‴), 4.25 (2H, H-2‴ and H-3‴), 4.27 (2H, H-2″ and H-4″), 4.35 (m, 1H, H-3″), 4.36 (m, 1H, H-6‴b), 4.38 (m, 1H, H-6″b), 4.44 (m, 1H, H-5″), 4.56 (m, 1H, H-6‴a), 4.81 (m, 1H, H-6″a), 5.08 (d, *J* = 7.8 Hz, 1H, H-1‴), 1H, 5.75 (d, *J* = 7.3 Hz, 1H, H-1″), 6.87 (d, *J* = 1.9 Hz, 1H, H-6), 7.09 (d, *J* = 1.9 Hz, 1H, H-8), 7.26 (d, *J* = 8.4 Hz, 2H, H-3′ and H-5′), 7.63 (d, *J* = 8.4 Hz, 2H, H-2′ and H-6′), 8.08 (s, 1H, H-2). ^{13}C NMR (pyridine-d_5) δ: 62.1 (C-6‴), 69.6 (C-6″), 70.5 (C-4″), 71.2 (C-4‴), 74.1 (C-2″), 74.7 (C-2‴), 76.9 (C-5″), 77.7 (C-3″)[a], 77.8 (C-3‴)[a], 77.9 (C-5‴), 94.7 (C-8), 101.1 (C-6), 101.3 (C-1″), 105.1 (C-1‴), 106.8 (C-4a), 115.6 (C-3′ and C-5′), 121.4 (C-1′), 123.4 (C-3), 130.4 (C-2′ and C-6′), 153.2 (C-2), 157.5 (C-4′), 158.6 (C-8a), 162.4 (C-5), 163.4 (C-7), 180.7 (C-4). [a]: interchangeable.

*2′-Hydoxy,5-methoxy Genistein-4′,7-O-diglucoside (**7**).* White solid; $[\alpha]_D^{22}$ −40.6° (*c* 0.1, 50%MeOH), UV(MeOH) λ_{max} 256 nm. HR-ESI-MS (negative) *m*/*z* 623.16312 $[M - H]^-$ (calcd for $C_{28}H_{31}O_{16}$ 623.16121). ^{1}H NMR (DMSO-d_6) δ: 3.18 (m, 1H, H-4″), 3.22 (m, 1H, H-2‴), 3.24 (m, 1H, H-3‴), 3.28 (m, 1H, H-2″), 3.29 (m, 1H, H-4‴), 3.31 (m, 1H, H-3″), 3.45 (4H, H-5″, H-5‴, H-6″b, and H-6‴b), 3.72 (2H, H-6″a and H-6‴a), 3.83 (s, 3H, C-5OC<u>H</u>$_3$), 4.82 (d, *J* = 7.5 Hz, 1H, H-1‴), 5.08 (d, *J* = 7.1 Hz, 1H, H-1″), 6.62 (d, 1H, *J* = 8.5 Hz, H-5′), 6.53 (s, 1H, H-3′), 6.61 (s, 1H, H-6), 6.74 (s, 1H, H-8), 7.03 (d, 1H, *J* = 8.5 Hz, H-6′), 8.04 (s, 1H, H-2). ^{13}C NMR (DMSO-d_6) δ: 56.1 (C-5O<u>C</u>H$_3$), 60.6 (C-6‴)[a], 60.7 (C-6″)[a], 69.6 (C-4‴)[b], 69.8 (C-4″)[b], 73.1 (C-3‴)[c], 73.2 (C-3″)[c], 76.6 (C-2″), 76.6 (C-2‴), 77.0 (C-5″)[d], 77.3 (C-5‴)[d], 95.6 (C-8), 97.2 (C-6), 99.9 (C-1″), 100.4 (C-1‴), 104.0 (C-3′), 106.6 (C-5′), 109.5 (C-4a), 113.3 (C-1′), 123.0 (C-3), 132.0 (C-6′), 152.3 (C-2), 156.4 (C-2′), 158.2 (C-4′), 158.8 (C-8a), 160.6 (C-5), 161.3 (C-7), 174.1 (C-4). [a, b, c, d]: interchangeable.

*2′-Hydroxy Genistein-4′,7-O-diglucoside (**8**).* White solid; $[\alpha]_D^{22}$ −61.6° (*c* 0.1, 50%MeOH), UV(MeOH) λ_{max} 256 nm. HR-ESI-MS (negative) *m*/*z* 609.14469 $[M - H]^-$ (calcd for $C_{27}H_{29}O_{16}$ 609.14556). ^{1}H NMR (DMSO-d_6) δ: 3.18 (m, 1H, H-4″), 3.23 (m, 1H, H-2‴), 3.34 (m, 1H, H-3‴), 3.26 (m, 1H, H-2″), 3.29 (m, 1H, H-4‴), 3.31 (m, 1H, H-3″), 3.45 (4H, H-5″, H-6″b, H-5‴, and H-6‴b), 3.72 (2H, H-6″a and H-6‴a), 4.82 (d, *J* = 7.4 Hz, 1H, H-1‴), 5.06 (d, *J* = 7.3 Hz, 1H, H-1″), 6.47 (d, *J* = 1.6 Hz, 1H, H-6), 6.55 (d, *J* = 8.3 Hz, 1H, H-5′), 6.59 (s, 1H, H-3′), 6.71 (d, *J* = 1.6 Hz, 1H, H-8), 7.11 (d, *J* = 8.3 Hz, 1H, H-6′), 8.29 (s, 1H, H-2). ^{13}C NMR (DMSO-δ_6) δ: 60.6 (C-6‴), 60.6 (C-6″), 69.6 (C-4‴), 69.6 (C-4″), 73.1 (C-3‴)[a], 73.2 (C-3″)[a], 76.4 (C-2‴)[b], 76.6 (C-2″)[b], 77.0 (C-5″)[c], 77.2(C-5‴)[c], 94.5 (C-8), 99.6 (C-6), 99.9 (C-1″), 100.4 (C-1‴), 103.9 (C-3′), 106.1 (C-4a), 106.6 (C-5′), 111.4 (C-1′), 120.5 (C-3), 132.1 (C-6′), 156.0 (C-2), 156.5 (C-2′), 157.3 (C-8a), 158.6 (C-4′), 161.6 (C-5), 162.9 (C-7), 180.5 (C-4). [a, b, c]: interchangeable

[^{3}H]DHT-AR in Vitro Binding Assay. This assay was performed according to the method described previously[11]. In brief, the gene sequence corresponding to the ligand-binding domain (AR-LBD, 609−919 a.a.) in the C-terminus of AR was expressed in the *E. coli* strain DH5α as a maltose-binding protein-fused protein (MBP-AR-LBD), followed by purification using amylose resin (BIO-RAD). Thus, the obtained recombinant MBP-AR-LBD (50 μg/mL), [^{3}H]-dihydrotestosterone (DHT, 2 nM), and test samples were incubated at 4 °C for 3 h. [^{3}H]DHT-bound MBP-AR-LBD was then precipitated with hydroxyapatite, and radioactivity was measured with a liquid scintillation counter. Values are the means of three independent determinations.

Detection of PSA mRNA by Real-Time RT-PCR. Prostate cancer (LNCaP) cells were incubated in RPMI 1640 medium supplemented with 2% charcoal-stripped serum for 24 h. Cells were then treated with DHT (0.1 nM) and test compounds. After 12 h, RNA from these cells was isolated, and the expression of PSA genes was determined by real-time quantitative reverse transcription-PCR (RT-PCR) and normalized to GAPDH mRNA. The primer sequences used were as follows: for PSA, 5′-AGG TCG GAG TCA ACG GAT TT-3′ (forward) and 5′-TAG TTG AGG TCA ATG AAG GG-3′ (reverse); for GAPDH, 5′-GGT CCT CAC AGC TGC CCA TC-3′ (forward) and 5′-CAG CCT GAG GCG TAG CAG GT-3′ (reverse). Values are the means of three independent determinations.

RESULTS

Isolation of Isoflavone Glucosides. Freeze-dried groundnuts (261.9 g) were pulverized in a mixer and extracted with MeOH. The filtrate was concentrated to dryness and washed with hexane. The insoluble precipitate (1.02 g) was applied on a silica gel column prepared and developed with CH_2Cl_2:MeOH:H_2O (3:1:0.1). From this chromatography, genistein was eluted at fraction 9−11. Fractions 15−70, which contain isoflavone glucosides as confirmed by TLC and HPLC analyses, were collected and further chromatographed with reversed phase HPLC using Developsil C30 developed with 17% CH_3CN. From this chromatography, pure compounds **1** (7.9 mg), **2** (5.4 mg), **3** (8.5 mg), **4** (3.2 mg), **5** (3.1 mg), and **6** (24.4 mg) were eluted at 18.3 min, 19.7 min, 28.1 min, 32.9 min, 34.4 min, and 40.3 min, respectively, and crude compounds **7** and **8** were eluted at 8.9 and 11.6 min, respectively. Crude compounds **7** and **8** were both purified by ODS HPLC developed with 20% MeOH (flow rate: 3.0 mL/min). The pure compounds were eluted at 15.0 min [**7** (3.2 mg)] and 16.8 min [**8** (3.6 mg)], respectively.

Structural Elucidations of Compounds 1−8. Compound **6** was identified as genistein-7-*O*-gentibioside by comparing MS, ^{1}H, and ^{13}C NMR data in pyridine-d_5 with those of reported data.[6]

The HR-ESI-MS(−) of **1** showed a $(M - H)^-$ peak at *m*/*z* 461.10949, and the molecular formula of **1** was determined as $C_{22}H_{22}O_{11}$ (calcd for $C_{22}H_{21}O_{11}$ 461.10839). ^{1}H,^{13}C NMR, ^{1}H−^{1}H DQF COSY, and HMQC spectral analyses of **1** clearly showed that the structure of **1** was constituted by O-substituted isoflavone (aglycone), one β-hexose ($J_{1,2}$ = 7.2 Hz), and one methoxy function (δ_H 3.83). In ^{1}H NMR, two meta-coupled (*J* = 2.0 Hz) sp^2 mehines were observed at δ_H 6.60 (H-6) and δ_H 6.72 (H-8), which are characteristic for 5-OH and 7-OH substitutions in the A ring. In addition, an sp^2 methine network of δ_H 6.91 (*J* = 8.2 Hz, H-6′) − δ_H 6.25 (*J* = 2.0, 8.2 Hz, H-5′) − δ_H 6.34 (*J* = 2.0 Hz, H-3′) was also observed in ^{1}H NMR, indicating a 2′,4′ or 3′,4′-*O*-substitution in the B ring. Furthermore, ^{1}H−^{13}C long-range couplings were observed from methoxy (δ_H 3.83) and H-6 to δ_C 160.6 (C-5), and from H-6′ to δ_C 123.3 (C-3) in the HMBC experiment. From these observations, the methoxy function at C-5 and 2′,4′-*O*-substitution in the B ring were confirmed, and the aglycone in **1** was determined to be 2′-hydroxy,5-methoxy genistein. In

^{1}H NMR, most signals derived from β-hexose were observed around the solvent peak (δ_H 3.30) under severe overwrapping, and we could not analyze their vicinal coupling constants. Thus, **1** was acetylated with Ac_2O/pyridine to give hexa-acetylated **1**. ^{1}H NMR and ^{1}H−^{1}H DQF COSY spectral analyses of hexa-acetylated **1** clearly proved the hexose to be β-glucose due to its large vicinal coupling constants [H-1′(δ_H 5.24, d, J = 7.2 Hz), H-2′(δ_H 5.31, dd, J = 7.2, 9.0 Hz), H-3′(δ_H 5.35, dd, J = 9.0, 9.3 Hz), H-4′ (δ_H 5.18, dd, J = 9.0, 9.3 Hz), H-5′(δ_H 3.96, ddd, J = 2.3, 5.6, 9.0 Hz), H-6′(δ_H 4.22, dd, J = 2.3, 12.3 Hz and δ_H 4.28, dd, J = 5.6, 12.3 Hz)]. The linkage of β-glucose at C-7 in aglycone was proven by the observations of ^{1}H−^{13}C long-range couplings from H-1′′ (δ_H 5.08), H-6, and H-8 to C-7 (δ_C 161.3) in the HMBC experiment. From the observations described above, **1** was identified as 2′-hydroxy,5-methoxy genistein-7-*O*-glucoside. Compound **1** was novel.

The HR-ESI-MS(−) of **2** showed a $(M - H)^-$ peak at m/z 609.14469, and the molecular formula of **2** was determined to be $C_{27}H_{30}O_{16}$ (calcd for $C_{27}H_{29}O_{16}$ 609.14556). ^{1}H,^{13}C NMR, ^{1}H−^{1}H DQF COSY, and HMQC (in DMSO) spectral analyses of **2** showed that the structure of **2** was constituted by an O-substituted isoflavone (aglycone) and two β-hexoses (each $J_{1,2}$ = 7.2 Hz). The ^{1}H and ^{13}C NMR signals derived from aglycone were similar between **1** and **2**, while the methoxy signal in **1** disappeared in **2**. Thus, the aglycone in **2** was determined to be 2′-hydroxy genistein. The two β-hexoses were confirmed to possess a gentibioside structure because the ^{1}H and ^{13}C NMR chemical shifts of the two hexose signals were completely identical to those of **6** in DMSO-d_6. The attachment of gentibioside at C-7 in aglycone was proven by the observations of ^{1}H−^{13}C long-range couplings from H-1′′ (δ_H 5.05), H-6 (δ_H 6.57), and H-8 (δ_H 6.82) to C-7 (δ_C 164.7) in the HMBC experiment. Thus, **2** was identified as 2′-hydroxy genistein-7-*O*-gentibioside. Compound **2** was novel.

The HR-ESI-MS(−) of **3** showed a $(M - H)^-$ peak at m/z 445.11528, and the molecular formula of **3** was determined to be $C_{22}H_{22}O_{10}$ (calcd for $C_{22}H_{21}O_{10}$ 445.11347). The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of **1**. Differences were observed only on the signals in the B ring. In ^{1}H NMR, the 2H equivalent methines of the B ring in **3** were observed as a doublet (J = 8.4 Hz) at δ_H 6.79 and δ_H 7.31 suggesting a 4′-*O*-substitution in the B ring. Considering these observations and the molecular formula of **3** (= **1** − O), **3** was identified as 5-methoxy genistein-7-*O*-glucoside.[12]

The HR-ESI-MS(−) of **4** showed a $(M - H)^-$ peak at m/z 475.12547, and the molecular formula of **4** was determined to be $C_{23}H_{24}O_{11}$ (calcd for $C_{23}H_{23}O_{11}$ 475.12404). The ^{1}H- and ^{13}C NMR spectra of **4** were similar to those of **1** and **3**. These signals derived from the B ring and the appearance of a methoxy signal (δ_H 3.78, δ_C 55.6) in **4** were different from **1** and **3**. The two doublet sp^2 methine signals, which coupled each other [H-5′ (δ_H 6.79, J = 8.2 Hz) and H-6′ (δ_H 6.90, J = 8.2 Hz)], and one singlet methine signal [H-2′ (δ_H 7.11)] were observed as B ring signals in the ^{1}H NMR spectrum of **4**, and ^{1}H−^{13}C long-range couplings were observed from H-2′ to C-6′ (δ_C 121.6) and C-3 (δ_C 124.9) in the HMBC spectrum of **4**. Therefore, a 3′,4′-O-substitution (B ring) in **4** was proven. In addition, ^{1}H−^{13}C long-range couplings from the methoxy (δ_H 3.78) and H-5′ to C-3′ (δ_C 147.0) were also observed, and the attachment of the methoxy at C-3′ was proven. From these findings, **4** was identified as 3′,5-dimethoxy genistein-7-*O*-glucoside. Compound **4** was novel.

The HR-ESI-MS(−) of **5** showed a $(M - H)^-$ peak at m/z 447.09314, and the molecular formula of **5** was determined to be $C_{21}H_{20}O_{11}$ (calcd for $C_{21}H_{19}O_{11}$ 447.09274). The ^{1}H and ^{13}C NMR signals of **5** derived from aglycone were identical to those of **2**, while the signals due to one β-glucose were observed in **5**. The attachment of β-glucose at C-7 was confirmed by the observations of ^{1}H−^{13}C long-range couplings from H-1′′ (δ_H 5.06) and H-6 (δ_H 6.46) to C-7 (δ_C 162.9). Thus, **5** was identified as 2′-hydroxy genistein-7-*O*-glucoside.[13]

The HR-ESI-MS(−) of **7** showed a $(M - H)^-$ peak at m/z 623.16188, and the molecular formula of **7** was determined to be $C_{28}H_{32}O_{16}$ (calcd for $C_{28}H_{31}O_{16}$ 623.16121). The ^{1}H signals of **7** derived from aglycone were closely similar to those of **1**, while low field shifts were observed at H-3′ and H-5′ (Δ0.27 and Δ0.19, respectively). In addition, the ^{1}H and ^{13}C signals due to two β-glucose units were observed in **7**. The attachment of the two β-glucoses was determined to be C-7 and C-4′ in aglycone because ^{1}H−^{13}C long-range couplings from H-1′′ (δ_H 5.08) and H-6 (δ_H 6.61) to C-7 (δ_C 161.3) and from H-1′′′ (δ_H 4.82) to C-4′ (δ_C 158.2) and NOE between H-1′′′ and H-5′ (δ_H 6.52) were observed in the HMBC and NOESY spectra of **7**, respectively. Thus, **7** was identified as 2′-hydroxy,5-methoxy genistein-4′,7-*O*-diglucoside. Compound **7** was novel.

The HR-ESI-MS(−) of **8** showed a $(M - H)^-$ peak at m/z 609.14469, and the molecular formula of **8** was determined to be $C_{27}H_{30}O_{16}$ (calcd for $C_{27}H_{29}O_{16}$ 609.14556).

The ^{1}H and ^{13}C NMR of **8** were similar to those of **7**. The only difference between **7** and **8** was the disappearance of methoxy signals in **8**. Therefore, **8** was identified as 2′-hydroxy genistein-4′,7-*O*-diglucoside.[14]

Effects of Compounds 1−8 on the Binding of DHT to AR. Compounds **2** and **5** inhibited the binding of DHT to AR in a dose-dependent manner, the IC_{50} values of **2** and **5** were 280 and 160 μM, respectively, and these inhibitory activities were about 4−7-fold less potent than that of flutamide,[15] which was clinically used for the treatment of prostatic diseases. However, compounds **1**, **3**, **4**, **6**, **7**, and **8** did not inhibit the binding of DHT to AR even at 400 μM. None of these compounds inhibited the binding of estradiol to the estrogen receptor up to 400 μM. (The binding inhibitory activities (IC_{50}) of genistein and daidzein for the estrogen receptor were 0.75 μM and 26 μM, respectively.) The above results are listed in Table 1.

Effects of Compounds 1−8 on DHT-Induced PSA Expression. Prostate-specific antigen (PSA) is a 33-kDa serine

Table 1. Effects of Compounds 1−8 on Binding to the Androgen Receptor (AR) and Estrogen Receptor (ER)

compd	AR	ER
	IC_{50} (μM)	IC_{50} (μM)
1	>400	>400
2	280	>400
3	>400	>400
4	>400	>400
5	160	>400
6	>400	>400
7	>400	>400
8	>400	>400
flutamide	39	>400
genistein	270	0.75
daidzein	>400	26

protease, whose expression in the prostate is triggered by the androgen-mediated action of AR. Therefore, to determine whether compounds **1**−**8** showed AR agonistic or antagonistic activity, we examined the effects of these compounds on the DHT-induced expression of PSA mRNA in prostate cancer LNCaP cells. As shown in Table 2, compounds **2** and **5**, which inhibited DHT binding to AR *in vitro*, suppressed the DHT-induced expression of endogenous PSA mRNA in LNCaP cells with IC_{50} values of 20 and 18 μM, respectively, whereas each compound alone failed to induce the expression of PSA mRNA (data not shown). These results showed that **2** and **5** possess AR antagonistic but not agonistic activities.

Table 2. Effects of Compounds 1−8 on DHT-Induced PSA Expression

compd	IC_{50} (μM)
1	>50
2	20
3	>50
4	>50
5	18
6	>50
7	>50
8	>50
flutamide	2.3

DISCUSSION

In this study, we succeeded in isolating 8 isoflavone glucosides (**1**−**8**) from the tubers of groundnut. From these compounds, **1**, **2**, **4**, and **7** were novel. Compounds **3**, **5**, and **8** have been reported to be included in the legume family such as *Ulex* and *Lupinus*,[12−14] with compound **6** being included in groundnut (*Apios*).[6] We could not find genistin, which was reported to be present in groundnut.[6]

We tested the binding activities of **1**−**8** for ER and AR and found that **2** and **5** inhibited DHT binding to AR but not estrogen binding to ER, indicating that these two compounds can bind to AR selectively. Furthermore, the activity of these compounds for AR was proven to be antagonistic with an assay using cultured LNCaP cells. Compounds **2** and **5** have been shown to be the first isoflavone glucosides to possess AR antagonistic activity.

AR, a member of the nuclear receptor superfamily, is a critical mediator of prostate cancer and benign prostatic hyperplasia; therefore, treatment with AR antagonists is expected to be an effective prostate cancer and benign prostatic hyperplasia therapy. The AR antagonist flutamide has been clinically used for prostate cancer therapy Thus, the development of a new type of AR antagonist is an attractive strategy to overcome these diseases.

We examined the structure−activity relationship of compounds **1**−**8** for AR antagonistic activity and concluded that the coexistence of the 2′, 4′-OH structure in the B ring and 5-OH in the A ring was important. Further biological studies on **1**−**8** are in progress.

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Notes

The authors declare no competing financial interest.

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